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76-48-FSL

BIODEGRADATION OF ALPHA TNT AND ITS PRODUCTION ISOMERS

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July 1975

UNITED STATES ARMY
NATICK RESEARCH and DEVELOPMENT COMMAND
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Food Sciences Laboratory

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High concentrations of cells of two pseudomonad isolates, IIBX, and S95YUN-5, effectively removed ^{14}C-labeled TNT from the reaction mixture, the ^{14}C-label being distributed between cells and filtrate. ^{14}C-activity was associated with all biochemical components of the cells obtained by solvent fractionation, and was found in chromatograms containing ninhydrin positive material from the hydrolysis of cellular proteins. Small amounts of nitrite were observed when the reaction was carried out in nitrogen-free medium, suggesting that some of the nitro groups may be released as nitrite.

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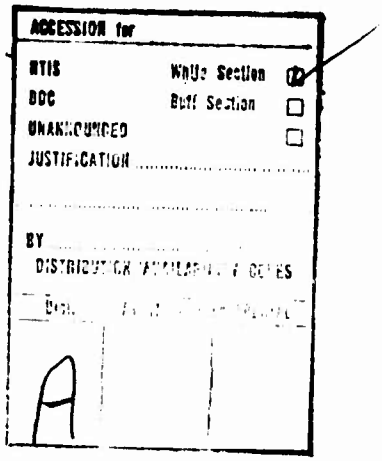
PREFACE

Wastes from the manufacture of, and shell loading of munitions contain sufficient concentrations of nitrobodies to constitute a potential toxic hazard. This study represents an effort to determine whether TNT degrading bacteria can be found which could be used to clean up these manufacturing wastes.



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I. Statement of the problem

Waste from the manufacture of and shell loading of munitions represents a serious disposal problem for the Department of Defense. In the manufacture of TNT, the plant wash water contains low quantities (50-100 ppm) of TNT and the waste from the Sellite Process contains TNT and the production isomers of TNT.

It has been shown that these nitrobodies can be adsorbed onto an XAD2 column and eluted from the XAD2 column with acetone or other organic solvents. This treatment may be part of an effective solution to this waste disposal problem in that it will generate a nitrobody free effluent water. The XAD2 system, however does not destroy nitrobodies, but results in a concentrated waste which must still be disposed of. The concentrated nitrobodies may be burned unless explosive dangers or serious air contamination problems are associated with the burning process, or if these nitrobodies are found to be biodegradable, they could be rendered ecologically safe by biological treatment.

II. Background

Recalcitrant molecules have specific structural characteristics which prevent microbial metabolism of the molecules, but at this time microbiologists are not able to describe all of these structural characteristics. Naturally occurring organic molecules are biodegradable by some microbial form (Dagley, 17), whereas, the truly recalcitrant molecules are found in broad classes of the synthetic organic compounds. In nature, many compounds which are potentially biodegradable, are slowly degradable or totally undegradable because of their interaction in the soil or the influence of environmental factors.

In studies of nitrphenols, Raymond and Alexander (1) isolated a bacterium able to metabolize p-nitrophenol and showed the appearance of stoichiometric amounts of nitrite in the medium and the formation of catechol. Tewfik and Evans (2) demonstrated the biodegradation of 3,5-dinitro-o-cresol via reduction of the 5-nitro group to an amino group which is then replaced by a hydroxyl group. After this the same series of reactions occur with the 3-nitro group. Ring fission occurs after the formation of the trihydroxyl species. The presence of at least two nitro grops onto the benzene ring did not necessarily confer recalcitrance on the molecule.

Moore (3) showed tha t a strain of Proactinomyces (Nocardia) was capable of sustaining growth on nitrobenzene as a sole source of carbon and nitrogen. Evans and Simpson (4) reported isolation of a pseudomonad capable of replacing the nitro group of either ortho- or para-nitrophenol with an hydroxyl group, resulting in nitrite liberation. Jensen and Gunderson (5) demonstrated that an atypical strain of Corynebacterium simplex, when growing on

dinitro-o-cresol, caused an increase in nitrite nitrogen in the medium corresponding to a decrease in substrate nitrogen. Gunderson and Jensen (5) observed degradation of several nitrophenols with a nitro group at the para position but no degradation of o-nitrophenol or of 2,5-dinitrophenol. The latter findings suggested the para-nitro group as susceptible to bacterial attack and, indeed, all of the nitrogen was released from p-nitrophenol but only 50% of the nitrogen was recovered as nitrite from 2,5-dinitrophenol (6).

One of the first serious studies of TNT biodegradation by microorganisms was reported by Osmon and Klausmeier (7). In their study, they tested for disappearance of TNT on agar plates containing 1% yeast extract and 1% glucose plus 100 µg of TNT. Their measure of degradation was the loss of TNT from the medium which can only be considered as presumptive evidence of degradation. In no case were they able to detect the disappearance of TNT in flasks containing only mineral salts and TNT.

A recent study by Won, et al., (8), described the metabolic disposition of TNT. They described the disappearance of TNT but also included in th eir media, glucose (0.1%), and yeast extract (0.5%). Suspensions of organisms consumed oxygen in the presence of TNT. Analysis of culture filtrates showed the conversion of TNT to 2,2',6,6'-tetranitro-4,4'-azoxytoluene, 2,2',4,4'-tetranitro-6,6'-azoxytoluene, 4-amino-2,6-dinitrotoluene, 2,6-dinitro-4-hydroxylaminotoluene, and 2-amino-4,6-dinitrotoluene. None of these intermediates indicate biodegradation of the TNT molecule, but only biotransformation of the ring substituted nitro groups.

A review of the literature points out that no laboratory working on TNT degradation has yet isolated an organism that will grow in a mineral salts medium with TNT present as the sole source of organic carbon and energy.

III. Materials and Methods

A. Inoculum preparation

Cell crops of isolates IIBX and S-9 were grown in 10 liters of Difco nutrient broth in a 20 liter carboy using a Chemap, Model E-1 Vibro-mixer fitted with a submersible spray pump. Acetate grown cells were cultured in M-9 (Traxler, 15) medium containing 0.4% (W/V) sodium acetate in a New Brunswick M-19

fermentor at 37C, stirred at 200 rpm and aerated at a rate of 1500 cc of compressed air per minute. The cells were collected after 24 hours incubation by batch centrifugation, washed 3X in 0.15 M NaCl containing 0.05 M $MgCl_2$ for cell stabilization, and resuspended in either M-9 or M-9 (-N) medium at the desired cell concentration.

B. Cellular Component Analysis

Figure 1 outlines the procedure used to separate cellular components into general classes of biochemical compounds on the basis of solubility. Our objective was not to fractionate the cell into as many separate components as possible but rather to achieve a system which would allow an estimate of ^{14}C-activity associated with the major classes of biochemical components. It must be understood, for example, that fraction S-2 contains primarily lipids and peptides but may also include other components which are soluble in this particular solvent system. It is also obvious that lipids will occur in other fractions such as the lipo-proteins present in the protein residue. The selection of a fractionation scheme is highly subjective because of the large number of available methods each of which has certain merits as well as certain defects. This method was chosen for its simplicity and the fact that it does provide a fair representation of major cellular components.

C. Protein Extraction and Hydrolysis

The method for obtaining cellular protein for the ^{14}C incorporation studies is outlined in Figure 2. This method consists of a cold TCA precipitation of protein from sonicated cells followed by dialysis of the protein against 0.05 M ammonium bicarbonate In some early experiments, ammonium sulfate was used for precipitation of the protein and dialysis was against 0.02 M phosphate buffer, pH 7.0. The protein was hydrolyzed with 6N HCl at 121C for 1 hour.

D. Chemical Determinations

In the determination of cellular components the following quantitative methods were used: Carbohydrates by anthrone, peptides and amino acids by the ninhydrin method, DNA by diphenylamine, RNA by the orcinol method, and protein by the Folin method for soluble proteins (16). Free nitrite in the culture medium was determined, after clarification, by the

Figure 1 Scheme for component analysis of cells.

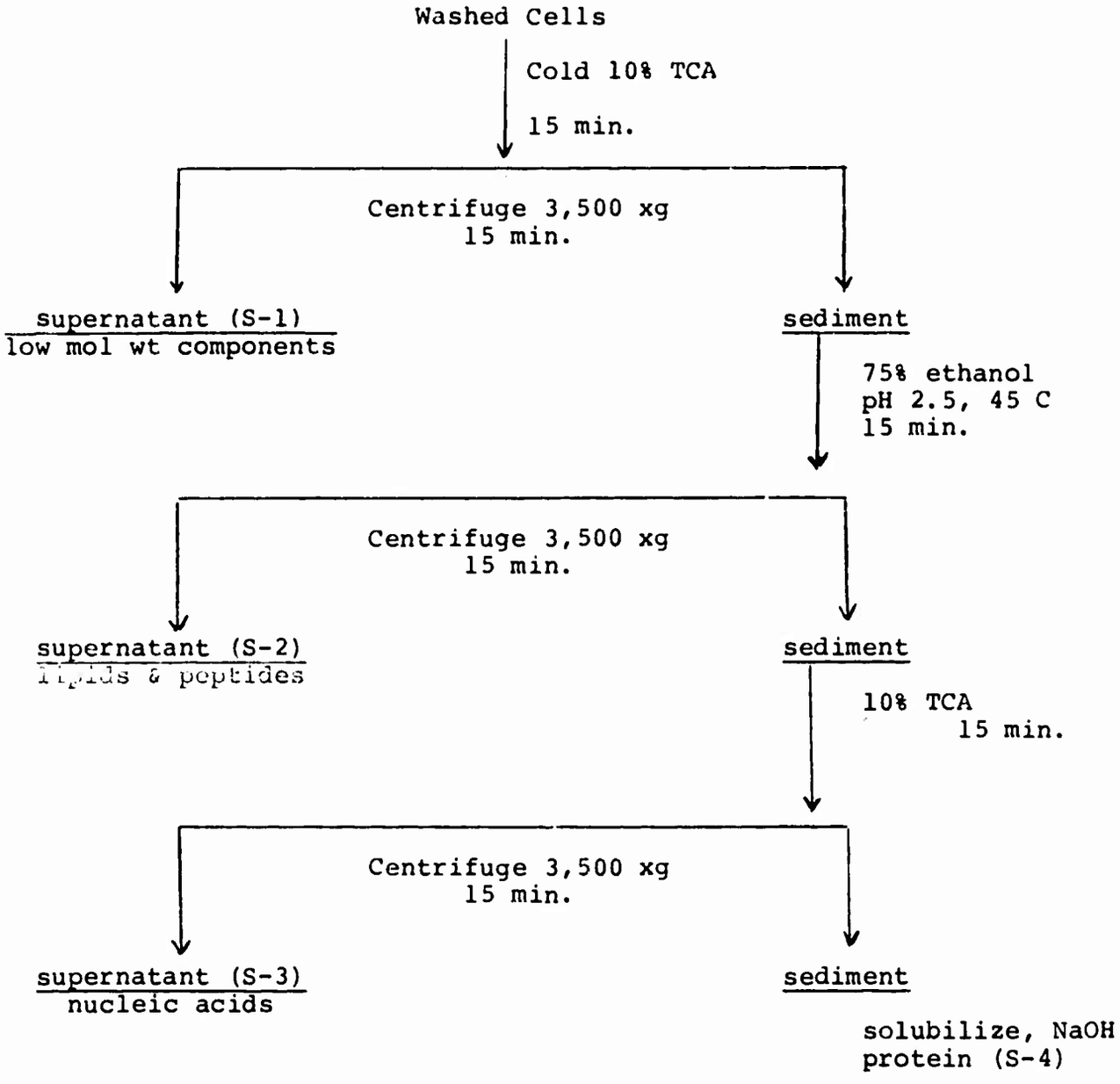
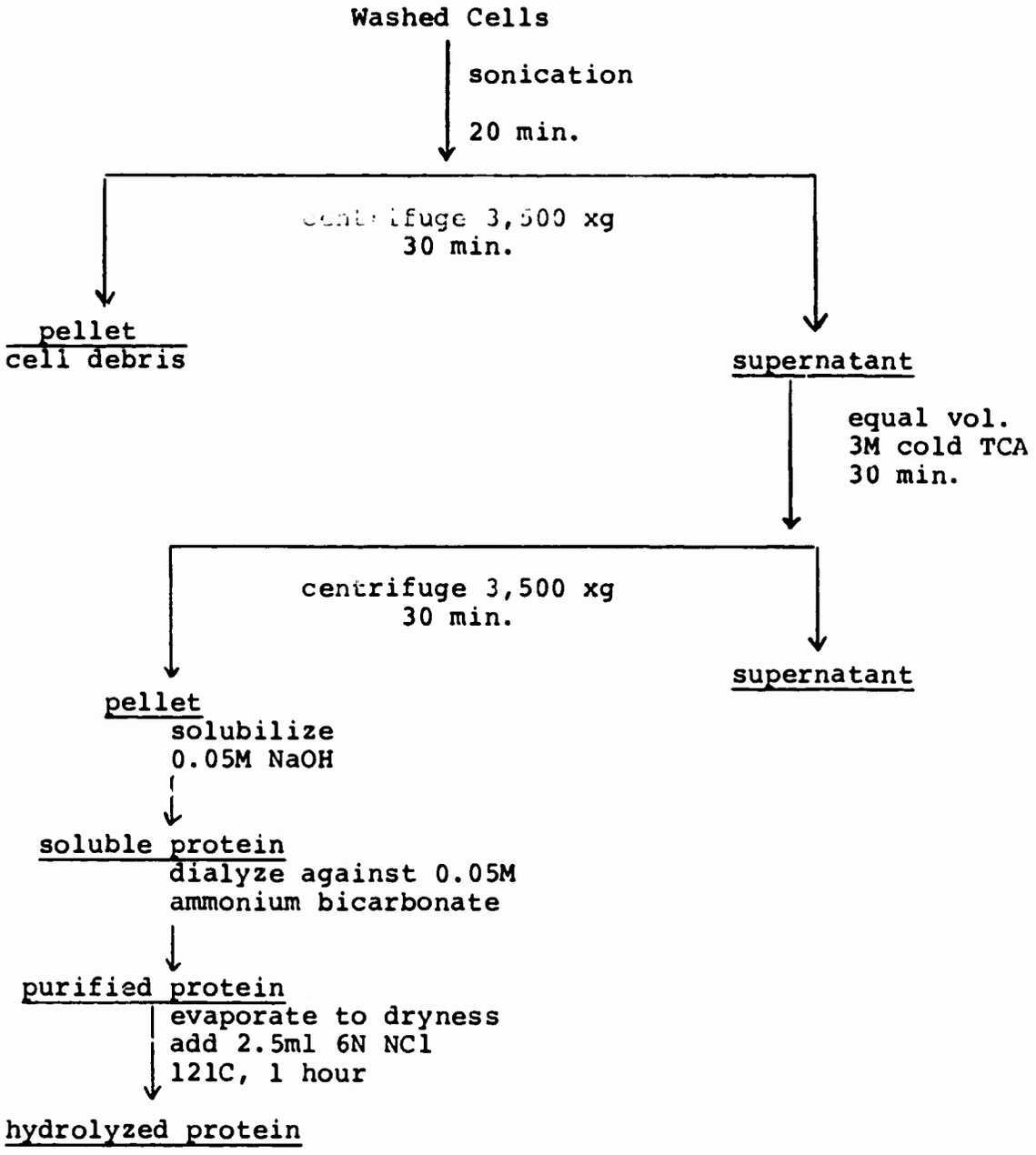


Figure 2 Recovery and hydrolysis of cellular protein.



sulfanilic acid-naphthylamine method. Ammonia production was measured using an Orion Ionalyzer (model 95-10) equipped with an ammonium ion specific electrode. The meter was calibrated at room temperature using a series of ammonium chloride standards. The ammonium ion concentration was determined in 50 ml of culture supernatant made alkaline (pH 11) by adding 1.0 ml of 10 N NaOH. The alkaline medium was agitated with a magnetic mixer to ensure adequate diffusion of ammonia gas across the probe membrane.

E. Dialysis Culture

The methodology and theories of dialysis culture are described by Humphrey (9). The dialysis culture used, supplied by Dr. J. M. Sieburth, Graduate School of Oceanography, University of Rhode Island, was of the type described in detail by Lavoie (10). The vessel consisted of two autoclavable plexiglass chambers separated by 90 mm diameter, 0.1 µM pore Nucleopore membrane. The medium chamber was equipped with a stirrer located immediately adjacent to the membrane to ensure constant diffusion rates across the membrane. An air sparger in the organism chamber aerated and agitated the cell suspension during incubation. The aeration rate was 1 liter/minute. The medium was recycled in a closed loop system via a peristal' : pump at a turnover rate of 1 hour.

The inoculum cells were grown in nutrient broth, harvested and washed 3X in 0.5 M tris buffer at pH 7.2, then incubated overnight in M-9 medium containing 100 µg/ml of TNT. The TNT adapted cells were harvested, washed 3X in tris buffer and resuspended in M-9 (-N) medium without TNT. The medium side of the dialysis culture vessel was charged with M-9 (-N) medium containing 100 µg/ml of TNT. Samples for TNT quantitation were taken from the recycling closed system just prior to entrance of the medium to the dialysis vessel. The entire culture device was maintained at 37C and sampled for a 72 hour period.

F. Chemicals

The chemicals and reagents used in this study were Mallinckrodt Analytical Reagent Grade or Fisher Reagent Grade or better quality. The solvents used in medium extractions, cell fractionations and thin layer or paper chromatography were Mallinckrodt Nanograde or Fisher Pesticide Grade. The (ring-UL-^{14}C) TNT was synthesized at the U. S. Army Development Center, Natick, Massachusetts by Dr. Ron Chalk.

IV. Results and Discussion

The capacity of large inocula to dissimilate alpha-TNT was studied during this phase of the investigation. Reaction flasks containing M-9 (-N) medium and 100 µg/ml of TNT were inoculated to give final cell concentrations of 7.5 to 34.7 µg/ml on a dry weight basis. At this cell concentration, there is no accurate method of measuring increase in cell numbers, nor would one expect growth to be likely, particularly with low substrate concentrations. The system was essentially a resting cell system, but it did afford an excellent means of studying the metabolic capability of the cells.

The necessity for yeast extract supplements with smaller inocula was a serious limitation to the overall objectives of this study. Yeast extract supplements could fulfill many functions such as providing required [illegible] or detoxification (Traxler, 11, 12). It might also substitute for cell division mediators which function in inoculum dependent lag effects in bacteria, yeasts, and other microorganisms.

Table 1. Effect of nutrient supplements on TNT disappearance from medium by a large inoculum of isolate IIBX.

Supplement	TNT Disappeared (%)
None (control)	94
Casamino acids, 100 µg/ml	97
Proteose peptone, 100 µg/ml	96
Yeast extract, 100 µg/ml	98
Vitamin mixture*	95

14 mg/ml cells, M-9 ($-NH_4^+$) + 100 µg/ml of TNT, 37C, 18 hours.

*2 µg/ml final concentration in medium of: riboflavin, thiamine, biotin, niacin, pyridoxine, and DL-desthiobiotin.

An experiment was designed to examine the effect of different nutrient supplements on TNT conversion with large inocula. Nutrient supplement, including yeast extract, had no significant stimulatory effect on TNT conversion by large inocula of isolate

IIBX (Table 1). The control flask, which consisted of unsupplemented M-9 (-N) medium containing 100 µg/ml of TNT, inoculated with 14 mg/ml of cells, and incubated for 18 hours, showed a disappearance of 94 percent of the TNT.

A high percentage of the TNT disappeared from the unsupplemented medium if the inoculum size was in excess of 7.5 mg of cells/ml (Table 2). An inoculum concentration of about 15 mg of cells/ml was adequate to achieve rapid (within 24 hours) disappearance of 90 percent of the TNT. Prolongation of incubation beyond 2 hours, or increases of cell mass beyond 15 mg/ml had no additional effect on alpha-TNT disappearance. It was not known from this experiment whether the initial rapid loss of TNT from the medium was due to adsorption to the cells, transport across the membrane, metabolic dissimilation or a combination of these factors.

Table 2. Effect of inoculum size on TNT removal from culture medium.

Cell Concentration (mg/ml)	Incubation time (hours)	TNT disappeared (%)
34.7	0	0
	2.0	88
	4.5	93
	22.0	94
14.8	0	0
	2.0	90
	4.0	93
	20.0	93
7.5	0	0
	2.0	16
	4.0	17
	20.0	21

Medium: M-9 ($-NH_4^+$), no yeast extract, 100 µg/ml of TNT.

The results in Table 3 describe the distribution of ^{14}C label from ring-UL-^{14}C TNT into cellular fractions and the culture filtrate by a large inoculum (13.5 mg/ml) of isolate IIBX. Of the initial $4.3x10^6$ dpm of radioactivity from substrate TNT, 53.5% of the radioactivity was recovered in the cells and 46.5% of the radioactivity was in the water soluble filtrate. Chemical analyses of the filtrate demonstrated 1,500 µg/ml of free amino acids and 95 µg/ml of free carbohydrate. The appearance of significant ^{14}C label in the filtrate containing this quantity of biochemical material suggests the secretion from the cells of amino acids and carbohydrates formed during the dissimilation of alpha-TNT.

Analysis of the culture filtrate from this experiment by thin layer chromatography (Figure 3) shows 18 ninhydrin positive spots, tentatively assumed to be amino acids, present in the filtrate. One-cm strips were cut from a duplicate chromatogram, scraped into scintillation vials and counted for ^{14}C activity. Radioactivity above the background level was present in each of the strips which contain ninhydrin positive material. The highest ^{14}C activity was found in the strips 12, 13 and 14 cm from the origin which contain the 4 ninhydrin positive areas indicated by asterisks in this figure. Additional studies indicate that aromatic transformation products could be present in this 3 cm portion of the chromatogram, yet there is radioactivity associated with the other sections of the chromatogram where only ninhydrin position material is found.

Figure 4 shows the distribution of ^{14}C activity and ninhydrin positive material found in a dialyzed protein extract (Figure 2) from isolate IIBX incubated with ^{14}C labeled TNT. The protein was hydrolyzed with 6N HCl for 1 hour at 121C. The hydrolysates were run on the TLC and cut down the center of each chromatogram. One section was sprayed with ninhydrin to develop the spots while the other side was cut into 1 cm strips for ^{14}C quantitation. The results indicate that ^{14}C from the TNT has been used in the synthesis of the amino acids incorporated into cellular protein since there is a correlation between ^{14}C activity and the ninhydrin positive material. The specific activity of the protein in this experiment was on the low side of the normal range, thus, in order to load sufficient hydrolysate for accurate dpm determinations, the quantity of amino acids was too high for satisfactory resolution by TLC. On-going experiments are being performed using protein hydrolysates with a slightly higher specific activity and the chromatography is performed by the paper method which allows for greater loading at the origin and resolution of individual amino acids.

Table 3. Component analyses and distribution of ring-UL-^{14}C-TNT in isolate IIBX.

Fraction	dpm	% Cellular Activity	% Original Activity
Whole cells	2.3×10^6	--	53.5
Culture filtrate (amino acids & carbohydrates)	2.0×10^6	--	46.5
Fraction S-1 (amino acids & carbohydrates)	3.2×10^5	14	7.4
Fraction S-2 (lipids & peptides)	3.5×10^5	15	8.1
Fraction S-3 (nucleic acids)	7.0×10^4	3	1.6
Cellular residue (protein)	3.0×10^5	13	7.0

13.5 mg cells/ml in 50 ml of M-9 (-N) medium supplemented with 100 µg/ml of TNT containing 4.3×10^6 dpm of ^{14}C activity. Incubated for 22 hours at 35C.

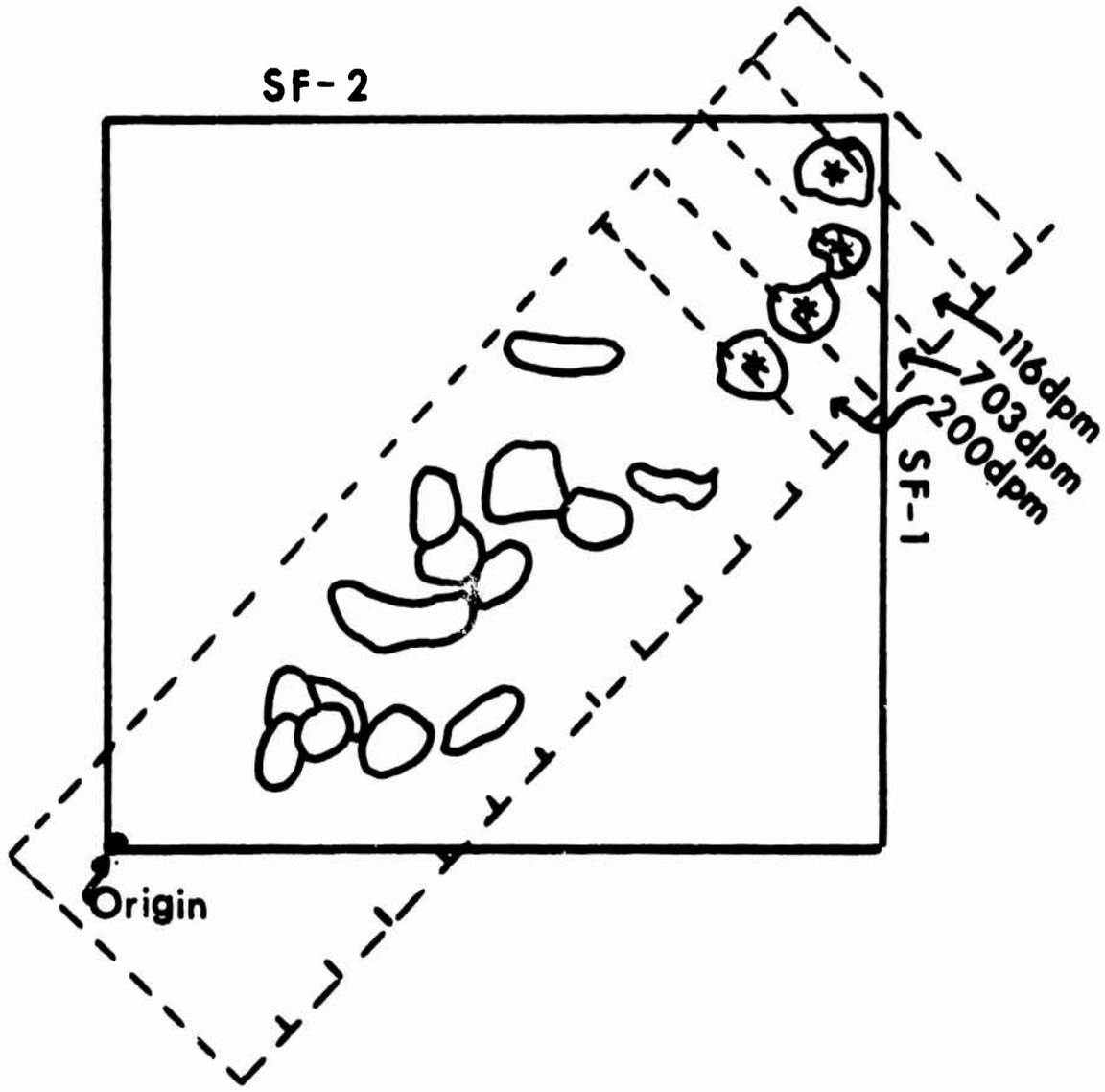


Figure 3. Two-dimensional thin layer chromatograph of culture filtrate developed with ninhydrin. Dpm determined from strips cut from a duplicate chromatograph. SF-1 (solvent front 1), isopropanol: formic acid: water, 7:1:2. SF-2, butanol: acetic acid: water, 4:2:1.

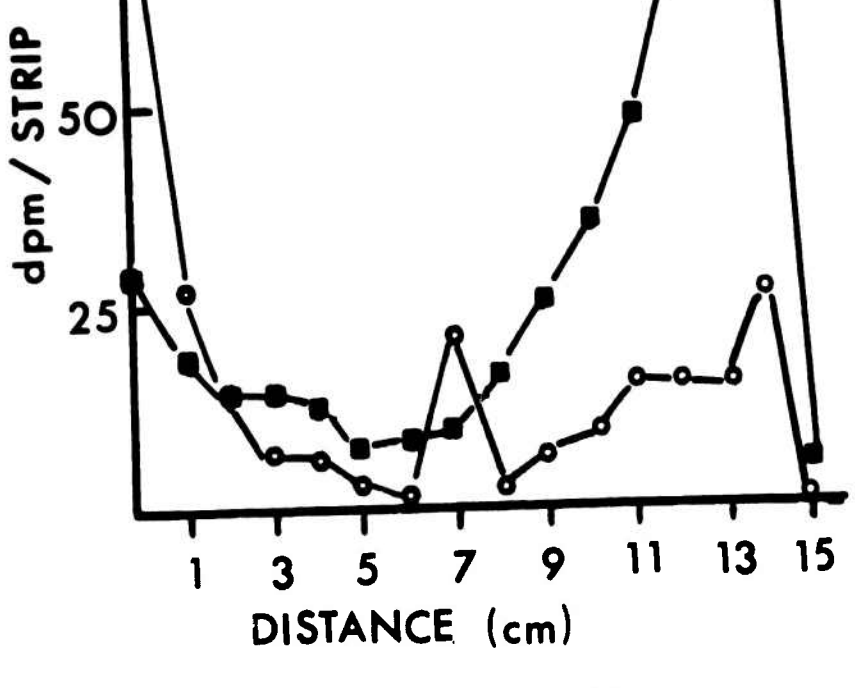


Figure 4. Ninhydrin positive area and ^{14}C activity of a TLC of a dialyzed protein hydrolysate from isolate IIBX incubated with TNT (ring-UL-^{14}C).

● TNT supplemented with 0.01% yeast extract.
■ TNT without yeast extract supplement.

Cellular incorporation of ^{14}C from TNT was also obtained using isolate S95YUN-5. The inoculum was divided into equal portions with one portion of the cell suspension heat killed at 70C for 30 minutes to serve as a control. The results in Table 4 show that 82.3% of the ^{14}C activity was recovered from cellular components of the live cell suspension but only 3.6% in the killed cell suspension. It appears that TNT adsorption by the cells and carry-over in the extraction process is a minor problem. The ^{14}C from TNT is found mainly in the lipid and peptides (Fraction S-2) of this isolate.

A modification of the ^{14}C incorporation study was performed using isolate S95YUN-5. In this experiment, concentration of cells was reduced and the reaction was run in a tightly closed vessel with a CO_2-free air sweep. The sweep air was carried from the reaction vessel to a methanol-ethanolamine trap to recover CO_2 evolved by the isolate during metabolism. The results in Table 5 demonstrate that ^{14}C from the TNT is distributed in the cells, CO_2 and culture supernatant. In this experiment, only 60.3% of the radioactivity was recovered.

The heterotrophic carbon dioxide fixation experiment described previously (15) was repeated with isolate S95YUN-5 (Table 6). Two concentrations of ^{14}C-sodium bicarbonate were used in this experiment, 0.22 and 0.022 mM, and the fate of ^{14}C was determined by measuring the amount of radioactivity in the washed cells after incubation. A quantitative test was run for residual TNT in the culture supernatant.

Carbon dioxide was not fixed by the heat killed cell suspension but was by the live cell suspension. It is interesting to observe that the live cell suspension supplemented with the higher level of sodium bicarbonate achieved complete TNT disappearance (within the limits of our assay).

Heterotrophic carbon dioxide fixation studies were run with isolates 12WT, S95YUN-5 and 125S at low inocula concentrations (Table 7). After incubation, the reaction mixture was acidified (pH 4.0) to release all unused bicarbonate as carbon dioxide which was flushed from the vessel by an air sweep. The ^{14}C content of the cells was interpreted as indicating the quantity of bicarbonate-carbon incorporated into cell bound organic constituents, whereas, that in the filtrate represented low molecular weight cellular constituents secreted into the medium. Considerable fixation occurred with all three strains but was the highest with isolate I25S, which also showed the

Table 4. Distribution of ^{14}C in the cellular components of isolate S95YUN-5 cultivated in modified M-9 medium containing (ring-UL-^{14}C) trinitrotoluene and yeast extract.

Fraction	Live cells dpm	Live cells % Total activity	Dead cells dpm	Dead cells % Total activity
S-1	$2.8x10^5$	9.32	$..2x10^4$	0.87
S-2	$2.1x10^6$	68.8	$1.3x10^4$	0.92
S-3	$3.8x10^4$	1.27	$3.0x10^3$	0.22
S-4	$8.6x10^4$	2.85	$2.3x10^4$	1.63

15 mg/ml cells, 37C for 20 hours in M-9(-N) medium containing 0.01% yeast extract, TNT 100 µg/ml ($3x10^6$ dpm of ring-UL-^{14}C). Dead cells heat killed, 70C for 30 mi tes.

Table 5. Distribution of ^{14}C by S95YUN-5 grown on UL-ring-^{14}C-TNT.

Location ^{14}C	dpm	% Initial activity	% Recovered activity
Initial TNT	$1.5x10^7$	--	--
Cells	$2.6x10^6$	17.3	28.7
Supernatant	$6.1x10^6$	40.7	67.5
CO_2 released	$3.5x10^5$	2.3	3.8
Total recovered	$9.1x10^6$	60.3	--

0.5 mg/ml cells, 37C for 20 hours in M-9(-N) medium containing 0.01% yeast extract, 100 µg/ml TNT ($1.5x10^7$ dpm of ring-UL-^{14}C).

Table 6. Heterotrophic carbon dioxide fixation by isolate S95YUN-5.

Sample	Live cells	Dead cells
A. .22 μM HCO_3^-		
Initial dpm	4.96×10^5	4.96×10^5
Cell bound dpm	5.7×10^3	4.0×10^2
% ^{14}C fixed	1.1	0.08
% TNT disappeared	84	0
B. .22 μM HCO_3^-		
Initial dpm	4.96×10^6	4.96×10^6
Cell bound dpm	3.8×10^4	6.0×10^2
% ^{14}C fixed	0.8	0.01
% TNT disappeared	100	0

Cells suspended in M-9(-N) containing unlabeled TNT, labeled $NaH^{14}CO_3$, incubated at 37C for 20 hours.

Table 7. Heterotrophic carbon dioxide fixation and TNT disappearance for three isolates.

Determination	[illegible]2NT	S95YUN-5	I25S
Klett Density, Inoculum	175	250	200
dpm x 10^4 in cells	4.78	3.58	5.69
dpm x 10^4 in filtrate	1.88	1.65	1.65
dpm total	6.66	5.23	7.34
CO_2 fixed, (%)	6.0	4.7	6.6
TNT used, (%)	[illegible]	45	75
Ratio $\frac{\text{TNT used}}{CO_2 \text{ fixed}}$	4.2	9.6	11.4

Initial $NaH^{14}CO_3$ was 1.11 dpm per flask, TNT 100 µg/ml in M-9 (-N) supplemented with 0.01% yeast extract, incubated at 37C for 20 hours.

greatest percentage of TNT lost from the medium. The percentage ratio of TNT used in the system to CO_2 fixed increased as the percentage of TNT used increased suggesting a fixed relationship between the two sources of carbon.

It was observed early in this investigation that if NH_4^+ was eliminated from the medium, the various isolates obtained better conversion of TNT. If the culture medium contains no added nitrogen, the only nitrogen sources available to the cells for growth or metabolism would be atmospheric N_2, amino acids secreted into the medium from the cells, or the ring bound nitro groups of TNT. If the ring bound nitro groups were removed from the ring (and replaced with -OH groups), they could be represented in the culture medium as nitrite ion. In order for nitrite to serve as an available source of nitrogen, it would have to undergo reduction via a mechanism similar to assimilative nitrate reduction, a known mechanism in other members of Pseudomonas. Samples of culture filtrate were analyzed for the presence of nitrite during growth of isolate S95YUN-5 on TNT in modified M-9 medium (Figure 5). Nitrite was detected in the medium and increased to a maximum value in this experiment at 6 hours incubation after which time the value remained constant for the remainder of the experiment. A qualitative test indicated that ammonium ion was present in the culture medium at the end of the experiment.

An experiment was designed to follow nitrite and ammonia levels in the medium under growth conditions as well as TNT disappearance (Figure 6). Nitrite accumulation in the medium followed the growth response of the organism; the break in the growth and nitrite curves corresponded closely to the break in TNT disappearance from the media. Ammonia in the medium increased to a maximum value at about 12 hours incubation, which was the time at which breaks occurred in the growth, nitrite accumulation and TNT utilization curves. Ammonia never accumulated in the medium to the level of nitrite, suggesting that ammonia is the form of nitrogen assimilated by the cells.

Table 8 is a tabulation of the ammonium ion, nitrite ion and TNT concentrations determined from the growth medium at various time intervals. The ammonia to nitrite-nitrogen ratio was 0.77 during the initial incubation period but decreased to 0.36 after 8 hours incubation. The ratio continued to decrease during the experiment to a low of 0.24. This relationship suggested that the reduction of nitrite to ammonia was a slower

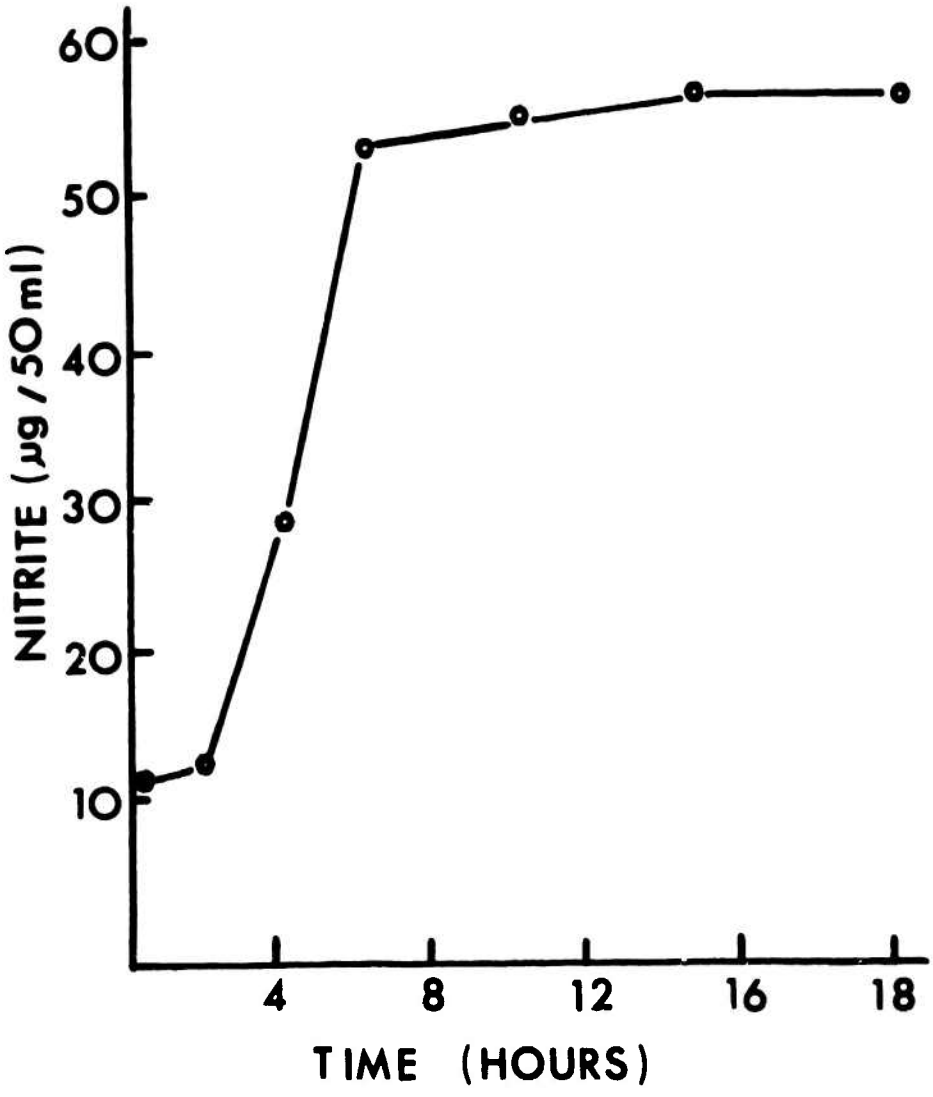


Figure 5. Nitrite released from TNT by isolate S95YUN-5 in ammonia free M-9 medium.

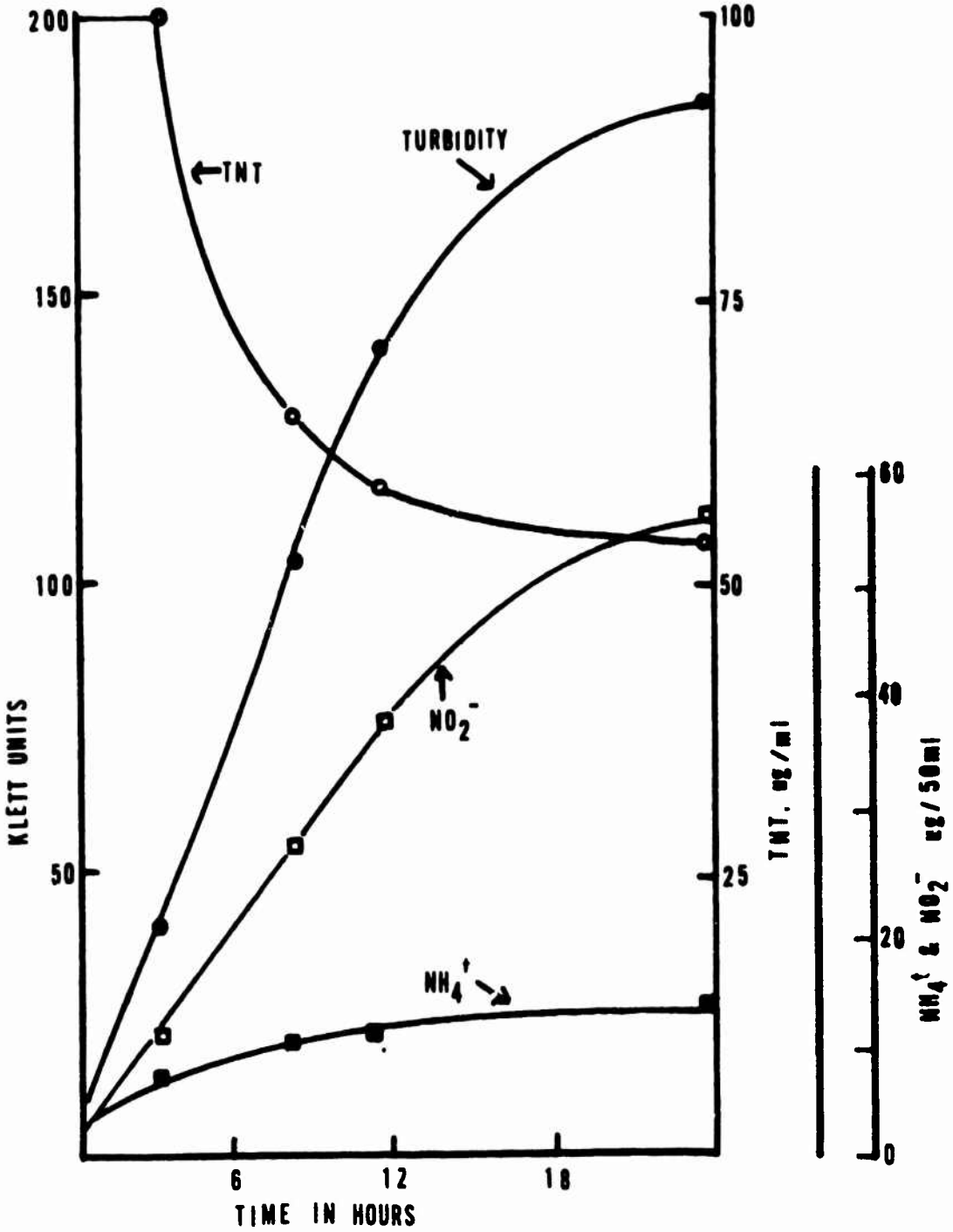


Figure 6. Relationships between turbidity, TNT loss, nitrite accumulation and ammonia accumulation.

reaction than the release of nitrogen from the ring or the cells use NH_4^+ almost as quickly as it is formed, therefore, it cannot accumulate. No method was devised to determine the rate or extent of nitrogen assimilated by the cells.

Table 3. Nitrite, ammonia and trinitriotoluene relationships during the growth of isolate S95YUN-5 in M-9(-N) medium containing TNT and yeast extract.

Time Hours	NH_4^+ µg/50ml	NO_2^- -N µg/50ml	$\frac{NH_4^+}{NO_2^- -N}$	TNT µg/ml
0	2.7	3.5	0.77	100
3	7.3	10.5	0.70	100
8	9.9	27.9	0.36	65
11	11.5	38.4	0.30	60
24	13.2	55.9	0.24	55

Initial TNT, 100 µg/ml; yeast extract 0.01%, incubated a shaking at 150 rpm.

The accumulation of nitrite and the presence of ammonia indicates the presence of an enzyme which functions in the removal of some of the nitro groups from TNT as well as a nitrite reductase system. Yamanaka (13) identified Pseudomonas cytochrome oxidase as a nitrite reducing system. Jurtshuk, Mueller and Acord (14) state that cytochrome [illegible] was induced when Pseudomonas aeruginosa was grown aerobically in the presence of nitrite. Oxygen tension exerts only minimal effects on cytochrome formation in Pseudomonas but lower oxygen tensions lead to maximum biosynthesis of the enzyme system components. With the inocula sizes used in these experiments, the aeration system cannot fully meet the oxygen demand, therefore, the oxygen tensions will be low and most favorable for induction of the nitrite reductase system.

The use of a "Dialysis" of "Diffusion" culture technique supported the data on dissimilation of TNT. The concept of dialysis culture is that if a bacterial culture and its substrate

are separated by a semipermeable membrane, the passage of nutrient across the membrane to the cells will follow the basic laws of diffusion. Very simply, if the substrate is not assimilated, the concentration of nutrient on both sides of the membrane will rapidly reach equilibrium. If the substrate is merely adsorbed the the cells, adsorption will occur and then an equilibrium will be established on the two sides of the membrane by the free substrate.

A dialysis culture experiment was performed with a 15 mg/ml suspension of isolate S95YUN-5 in M-9(-N) medium. One run contained viable cells suspended in this medium and a second run contained cells heat killed at 70C for 30 minutes. In both cases, the reservoir side of the dialysis culture vessel was charged with M-9(-N) medium supplemented with 100 µg/ml of TNT. Yeast extract was not used in this experiment. The medium was recycled through the reservoir system and sampled at intervals just prior to reentry to the dialysis chamber. Figure 7 shows that the concentration of TNT in the vessel containing live cells follows a first order decay curve throughout the 70 hours of incubation with no evidence of a TNT equilibrium being established between the two sides of the membrane. The vessel containing heat killed cells shows a slow decrease in TNT concentration which stabilized indicating a TNT equilibrium between the two sides of the membrane.

These dialysis experiments will be repeated and will be modified to study reaction products in each side of the reaction vessel. This system shows promise as a mechanism for studying the mechanisms involved in TNT loss from the culture system.

V. Conclusions

1. High cell concentrations effectively remove TNT from culture media and do not require a yeast extract supplement to be effective.

2. ^{14}C from (ring-UL-^{14}C) TNT is distributed between the cells and culture filtrate with large inocula of isolate IIBX and S95YUN-5.

3. ^{14}C-activity is associated with all biochemical components of the cells obtained by solvent fractionation.

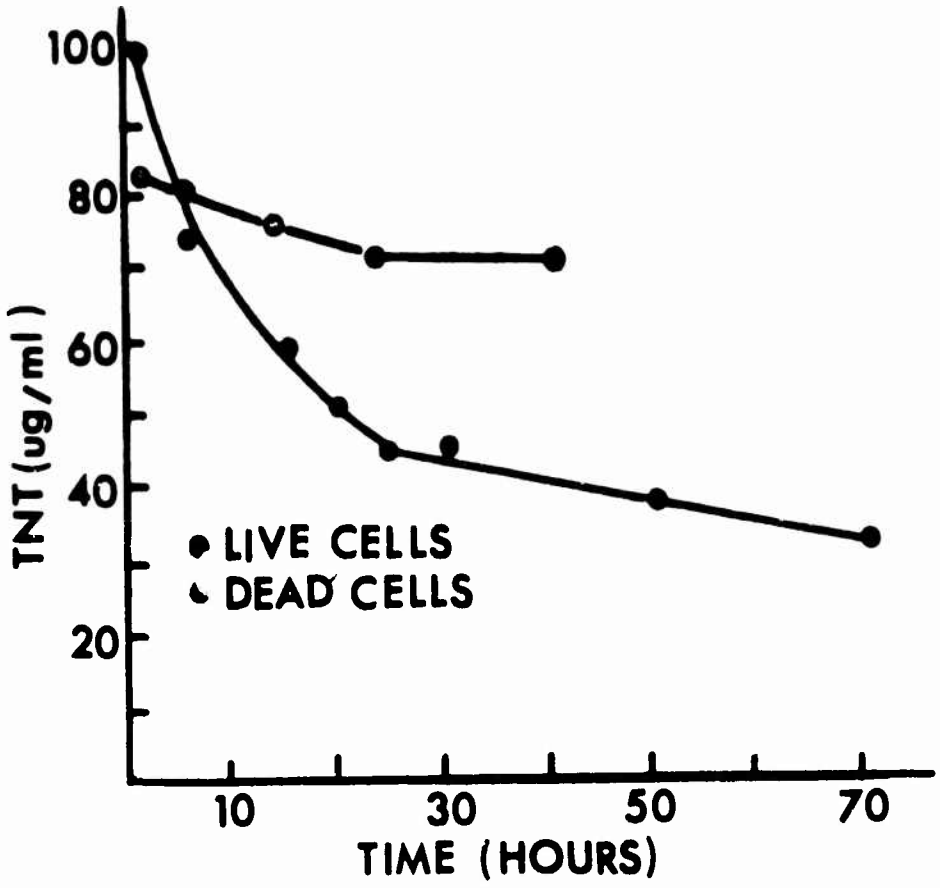


Figure 7. Loss of TNT from culture medium in a dialysis culture vessel.

4. ^{14}C-activity has been found in chromatographs which contain ninhydrin positive material obtained from the hydrolysis of cellular protein.

5. Heterotrophic carbon dioxide fixation has been demonstrated with isolates I2WT, S95YUN-5 and I25S. The ^{14}C from bicarbonate is found in cells incubated with TNT and low molecular weight water soluble compounds in the culture filtrate. There is a definite relationship between the amount of TNT lost from the culture and the amount of carbon dioxide fixed by the cells.

6. Experiments in ammonia-free culture medium demonstrated that the nitro groups on the TNT were released as nitrite ion which is reduced to ammonia for assimilation by the cells.

7. Dialysis culture studies with isolate S95YUN-5 against 100 µg/ml of TNT showed a first order decay curve for TNT in solution. The lack of an established equilibrium over a 70 hour incubation period indicated possible TNT metabolism by the isolate.

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